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Document Title
MUTAGENICITY EVALUATION OF FHP 3002 LIQUID PREPOLYMER IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY (FINAL) WITH COVER LETTER

Chemical Category
TOLUENE DIISOCYANATE (26471-62-5)

CONTAINS NO CBI

GRACE 86-910000057

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January 16, 1991

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Attn: Health and Safety Reporting Rule (N ification/Reporting)

Please find attached 8(d) health safety reports for mixtures processed containing toluene diisocyanate (CAS #26471-62-5), 4,4-Diphenylmethane diisocyanate (CAS #101-68-8) and 1,6-Diisocyanatohexane (CAS #822-06-0). Grace is submitting these reports for late filing since their submittal may have been subject to the isocyanates 10-year call-in of June 1, 1987.

We have reason to believe that some of these reports may have previously been submitted to EPA as attachments to PMN submissions. However, Grace is filing them as a precautionary measure to insure EPA's receipt.

These reports are being submitted for:

W. R. Grace & Co.-Conn.
Washington Research Center
7379 Route 32
Columbia, MD 21044

Sincerely,

J. W. Raksis

A:\JR91-013/lw

Attachments - 20

86910000638

FHP 3002 Prepolymer

CONTAINS NO CBI

GENETICS ASSAY NO. 5124

LBI SAFETY NO. 6008

TOLUENE DIISOCYANATE
26471-62-5

1,6- DIISOCYANATO HEXANE
822-06-0

MUTAGENICITY EVALUATION OF FHP 3002 (11601-11) LIQUID PREPOLYMER

IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

W.R. GRACE AND COMPANY
7379 ROUTE 32
COLUMBIA, MARYLAND 21044

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20989

REPORT DATE: OCTOBER, 1980

Litton BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I - IX. Items I - IV provide sponsor and compound identification information, type of assay, and the assay design reference number. All assay design references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled Assay Design, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.

I. SPONSOR: W.R. Grace and Company

II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 5124

A. Identification: FHP 3002 (11601-11) Liquid Prepolymer

B. Date Received: May 15, 1980

C. Physical Description: Viscous pale yellow liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. ASSAY DESIGN NUMBER: 431

V. STUDY DATES:

A. Initiation: May 21, 1980

B. Completion: September 22, 1980

VI. SUPERVISORY PERSONNEL:

A. Study Director: Brian C. Myhr, Ph.D.

B. Laboratory Supervisor: Jane Fisher

VII. RESULTS:

The data are presented in Tables 1 to 3 on pages 6 to 8.

VIII. INTERPRETATION OF RESULTS:

The test material, FHP 3002, Liquid Prepolymer, was immiscible with deionized water at 100 µl/ml but dissolved in dimethylsulfoxide (DMSO) at concentrations at least as high as 400 µl/ml. DMSO was chosen as the vehicle for the study and stock solutions were prepared just prior to each testing purpose by serial dilutions with DMSO. Treatments were initiated by diluting the stocks 1:100 into tubes of culture medium containing the cells. The test material precipitated in the culture medium for concentrations exceeding about 30 nl/ml. Increasing cloudiness with fine white droplets and solid material were observed as the concentration was increased; large amounts of polymerized solid were obtained at concentrations exceeding about 1500-2000 nl/ml.

The preliminary cytotoxicity test was performed over the concentration range of 1000 nl/ml to 1.95 nl/ml. Essentially no toxicity to cell growth was observed twenty-four hours after the treatments. Thus, in an attempt to achieve some toxicity and extend the assay to excessive concentrations, a series of applied concentrations up 4000 nl/ml (4 µl/ml) was used in the subsequent mutation assays.

VIII. INTERPRETATION OF RESULTS (continued):

Four trials of the mutation assay were initiated, although the last trial was performed with S9 activation only. The first trial was lost to an incubator over-heating problem and no mutation data was collected. The results of the other three trials are presented in Tables 1 to 3.

Under nonactivation conditions, no significant increases in mutant frequency were observed in the first trial (Table 1) for treatments with concentrations from 125 nl/ml to 1500 nl/ml. The minimum criterion for indicating mutagenesis was a mutant frequency exceeding 44.2 x 10^{-6}; this value was not approached in any of the treated cultures. The mutant selection dishes were lost to mold contamination for the 2000 nl/ml treatment, so a mutant frequency could not be calculated. The toxicity of treatment was highly variable and not related to the applied concentration. Thus, moderate toxicity was observed at 125 nl/ml and 1000-2000 nl/ml, but the 250 and 500 nl/ml treatments had very little or no effects on the cells. Doses higher than 2000 nl/ml were not analyzed because these treatments also had very little effect on cell growth. The variable toxicity was probably caused by the precipitation and polymerization of the test material, leading to nonhomogeneous contact between the cells and portions of the test material available to interact with the cells. The results of this trial strongly indicated the lack of mutagenic activity, but due to a subnormal average cloning efficiency for the negative controls (63%), another trial at higher cloning efficiency and higher doses was desirable to increase confidence in this conclusion.

The results of the next nonactivation trial (Table 2) were obtained with a normal negative control cloning efficiency of 71% and were confirmatory to the earlier trial. Concentrations of test material from 500 nl/ml to 4000 nl/ml did not induce mutant frequencies that exceeded the criterion of 33.0 x 10^{-6} used to indicate mutagenic activity in this trial. A mutant frequency could not be calculated for the 2000 nl/ml treatment because of the loss of the viable colony dishes, but the total number of mutant clones was similar to the numbers obtained for the other treatments, so no increase in frequency would have been expected. The treatments were only weakly toxic in this trial, but excessive amounts of precipitated material made higher doses impractical. Thus, the test material was evaluated as nonmutagenic under nonactivation conditions in this mutation assay system.

In the presence of the S9 activation mix, the toxicity of the test material remained substantially the same as without the mix, and large variations in toxicity as a function of applied concentration continued to occur. The mutant frequencies in the

VIII. INTERPRETATION OF RESULTS: (continued)

cultures exposed to a concentration range of 250 nl/ml to 2500 nl/ml remained strictly comparable to the negative control values in the first trial (Table 1). No indication of mutagenic activity was therefore obtained. However, the average cloning efficiency for the negative controls was only 54%, which just met assay acceptance criteria for supportive information. Another trial became necessary to exclude the possibility that the cloning conditions were too poor to allow the growth and detection of newly-induced mutants.

Small increases in the mutant frequency were observed in the second activation trial (Table 2) along with a highly unusual large increase in the culture exposed to 3000 nl/ml. The minimum criterion for indicating mutagenesis by a given treatment was a mutant frequency greater than 37.2×10^{-6}. The treatments with 1000 nl/ml and 2000 nl/ml resulted in mutant frequencies above this value and representing about 2-to 3-fold increases over the background (average of the solvent and untreated negative control values). A large, 9.7-fold increase was observed at 3000 nl/ml. A technical deficiency responsible for this sudden increase, unsupported by similar increases at lower and higher doses, was not apparent. Although the magnitude of this increase was of doubtful reliability, the result was interpreted as at least indicating a significant increase in mutant frequency. Another trial was performed to confirm the observed increases.

The third activation trial (Table 3) provided additional evidence for mutagenic activity but in a manner extremely difficult to interpret. A large increase in mutant frequency (8.6-fold over background) was again observed for the 3000 nl/ml treatment but not for other similar treatments. In this trial as well as Trial 2, the number of mutant colonies was clearly elevated and no contribution to the colony count from the carry-over of precipitated test material was evident. The magnitude of this response was not interpretable beyond the fact that a significant increase in mutant frequency was obtained. A smaller increase (about 2-fold over background) was also observed for the 3500 nl/ml treatment, where the mutant frequency just exceeded the criterion of 75.7×10^{-6} used to indicate a significant response in this trial. The general lack of correlation between the mutant frequency increases and the toxicity or applied concentration made interpretation of the activation assay data uncertain. This variability was probably caused by the extremely nonhomogeneous treatment conditions. The fact that several increases were observed in two trials argues for the existence of some mutagenic activity. This activity was considered to be weak because a clear response pattern was not demonstrated and the magnitudes of the large, isolated increases in mutant frequency were not interpretable.

VIII. INTERPRETATION OF RESULTS: (continued)

The average cloning efficiencies for the negative controls varied from 63% (Table 1) to 71% (Table 2) without activation and from 85% (Table 2) to 75% (Table 3) with activation, which demonstrated good-to-sufficient culturing conditions for these assays. The low cloning efficiency for the activation assay in Table 1 has already been noted. The negative control mutant frequencies were all in the normal range, and the positive control compounds induced mutant frequencies that were greatly in excess of the backgrounds. A reduced S9 activation efficiency was indicated in Table 2 by a DMN-induced mutant frequency below the normal lower limit of 200×10^{-6}, but mutagenic activity by the test material was still observed under the test conditions.

IX. CONCLUSIONS:

The test material, FHP 3002, Liquid Prepolymer, induced significant increases in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells only in the presence of rat liver S9 metabolic activation. Without activation, applied concentrations up to 4000 nl/ml caused variable toxicity but did not induce increases in the mutant frequency. With activation, 2-to 3-fold increases were observed in the 1000 nl/ml to 3500 nl/ml concentration range in a manner not related to toxicity or applied concentration. In addition, an unusual and large increase occurred at 3000 nl/ml in two trials. These results were interpreted as evidence for weak mutagenic activity in the Mouse Lymphoma Forward Mutation Assay with S9 metabolic activation.

Submitted by:

Study Director

Brian C. Myhr 10/28/80

Brian C. Myhr, Ph.D. date
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

James L. Liverman 10/28/80

David J. Brusick, Ph.D. Ph.D date
Director
Department of Genetics
and Cell Biology

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: LIQUID PREPOLYMER FHP 3002 11601-11
B. LBI CODE #: 5124
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 07/28/80

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
NONACTIVATION											
SOLVENT CONTROL	---	---	11.2	7.3		100.0	34.0	200.0	100.0	100.0	17.0
SOLVENT CONTROL	---	---	9.2	9.6		100.0	39.0	174.0	100.0	100.0	22.4
UNTREATED CONTROL	---	---	9.0	6.0		63.5	56.0+	194.0	103.7	65.9	23.9
EMS .5 UL/ML	---	---	8.5	6.9		69.0	560.0	156.0	83.4	57.5	359.0
TEST COMPOUND											
125.000 NL/ML	---	---	4.0	8.0		37.6	59.0	201.0	107.5	40.4	29.4
250.000 NL/ML	---	---	7.7	9.6		86.9	40.0	252.0	134.8	117.1	15.9
500.000 NL/ML	---	---	7.9	9.0		83.6	69.0	341.0	182.4	152.5	20.2
1000.000 NL/ML	---	---	3.3	6.6		23.3	71.0+	206.0	110.2	25.6	34.5
1500.000 NL/ML	---	---	3.3	13.2		46.6	62.0	219.0	117.1	54.5	28.3
2000.000 NL/ML	---	---	3.4	9.0		31.7	c	174.0	93.0	29.5	--
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	6.2	6.3		100.0	109.0	184.0	100.0	100.0	59.2
SOLVENT CONTROL	RAT	LIVER	6.8	8.1		100.0	68.0	158.0	100.0	100.0	43.0
UNTREATED CONTROL	RAT	LIVER	6.1	8.9		115.3	67.0	147.0	86.0	99.2	45.6
DMN .3 UL/ML	RAT	LIVER	4.0	5.1		43.3	216.0	53.0	31.0	13.4	407.5
TEST COMPOUND											
250.000 NL/ML	RAT	LIVER	5.4	10.4		119.3	89.0+	162.0	94.7	113.0	54.9
500.000 NL/ML	RAT	LIVER	1.9	5.7		36.3	81.0	172.0	100.6	36.5	47.1
1000.000 NL/ML	RAT	LIVER	2.2	9.0		57.4	101.0	192.0	112.3	64.4	52.6
2000.000 NL/ML	RAT	LIVER	1.1	2.9++		18.5	117.0	220.0	133.1	24.6	53.2
2500.000 NL/ML	RAT	LIVER	4.4	10.1		94.4	93.0	155.0	90.6	85.6	60.0

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100.
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4. THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4. THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
\+ ONE PLATE CONTAMINATED: VALUE BASED ON REMAINING TWO PLATES.
c TWO OR MORE PLATES CONTAMINATED: NO VALUE CALCULATED.
++ DUE TO TOXICITY, LESS THAN 3.0 x 10^6 CELLS WERE AVAILABLE FOR CLONING: THE ENTIRE CULTURE WAS ASSAYED AND 290 CELLS WERE SEEDED FOR VIABLE CLONE COUNT. VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

1. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: LIQUID PREPOLYMER FHP 3002 11601-11
B. LBI CODE #: 5124
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 08/18/80

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
NONACTIVATION											
SOLVENT CONTROL	---	---	13.2	7.7		100.0	32.0	216.0+	100.0	100.0	14.8
SOLVENT CONTROL	---	---	10.2	7.1		100.0	45.0	216.0+	100.0	100.0	20.8
UNTREATED CONTROL	---	---	10.1	9.4		109.1	21.0	203.0	94.0	102.5	10.3
EMS .5 UL/ML	---	---	5.9	6.2		42.0	348.0	86.0+	39.8	16.7	404.7
TEST COMPOUND											
500.000 NL/ML	---	---	10.5	6.8		82.0	33.0	222.0+	102.8	84.3	14.9
1000.000 NL/ML	---	---	9.9	10.0		113.8	44.0	162.0	75.0	85.3	27.2
2000.000 NL/ML	---	---	8.3	7.0		66.8	47.0	C	---	---	---
3000.000 NL/ML	---	---	7.6	9.2		80.3	63.0	254.0+	117.6	94.5	24.8
4000.000 NL/ML	---	---	6.6	12.2		92.5	28.0	192.0+	88.9	82.2	14.6
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	11.1	12.7		100.0	39.0	C	---	---	---
SOLVENT CONTROL	RAT	LIVER	8.9	12.3		100.0	47.0	276.0+	100.0	100.0	17.0
UNTREATED CONTROL	RAT	LIVER	9.3	11.1		82.4	45.0	235.0	85.1	70.2	19.1
DMN .3 UL/ML	RAT	LIVER	7.3	6.6		38.5	148.0	90.0+	32.6	12.6	164.4
TEST COMPOUND											
500.000 NL/ML	RAT	LIVER	10.3	10.3		84.7	78.0	251.0+	90.9	77.0	31.1
1000.000 NL/ML	RAT	LIVER	8.5	12.3		83.5	74.0	193.0	69.9	58.4	38.3
2000.000 NL/ML	RAT	LIVER	19.1	5.3		80.8	96.0	175.0	63.4	51.2	54.9
3000.000 NL/ML	RAT	LIVER	5.5	7.1		31.2	324.0	185.0+	67.0	20.9	175.1
4000.000 NL/ML	RAT	LIVER	5.0	10.6		42.3	76.0	210.0	76.1	32.2	36.2

* (RELATIVE SUSPENSION GROWTH x RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
\+ ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.
C TWO OR MORE PLATES CONTAMINATED; NO VALUE CALCULATED.

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 3

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: LIQUID PREPOLYMER FHP 3002 11601-11
B. LRI CODE #: 5124
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 09/10/80

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	6.5	10.8		100.0	106.0	218.0	100.0	100.0	48.6
SOLVENT CONTROL	RAT	LIVER	8.0	13.0		100.0	56.0	217.0	100.0	100.0	25.8
UNTREATED CONTROL	RAT	LIVER	7.5	10.7		92.1	136.0	238.0	109.4	100.8	57.1
DMN .3 UL/ML	RAT	LIVER	4.9	6.0		33.8	99.0	30.0	13.8	4.7	330.0
TEST COMPOUND											
250.000 NL/ML	RAT	LIVER	5.0	8.2		47.1	119.0	182.0	83.7	39.4	65.4
500.000 NL/ML	RAT	LIVER	5.8	6.0		40.0	60.0	202.0	92.9	37.1	29.7
1000.000 NL/ML	RAT	LIVER	6.3	5.0		36.2	56.0	176.0	80.9	29.3	31.8
2000.000 NL/ML	RAT	LIVER	1.7	6.3		21.7	75.0	110.0	50.6	11.0	68.2
3000.000 NL/ML	RAT	LIVER	1.0	3.8+++		13.1	311.0	107.0	38.8	5.1	290.7
3500.000 NL/ML	RAT	LIVER	1.1	6.0		20.7	120.0	155.0	71.3	14.7	77.4

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
+++CULTURE NOT SPLIT BACK TO 3.0 X 10^6 CELLS; THE ENTIRE CULTURE WAS ASSAYED AND 380 CELLS SEEDED FOR VIABLE CLONE COUNT.
VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

ASSAY DESIGN (NO. 431).

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.

3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 µl/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 µl/ml as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.

B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 µl S9/ml.

3. S9 Homogenate

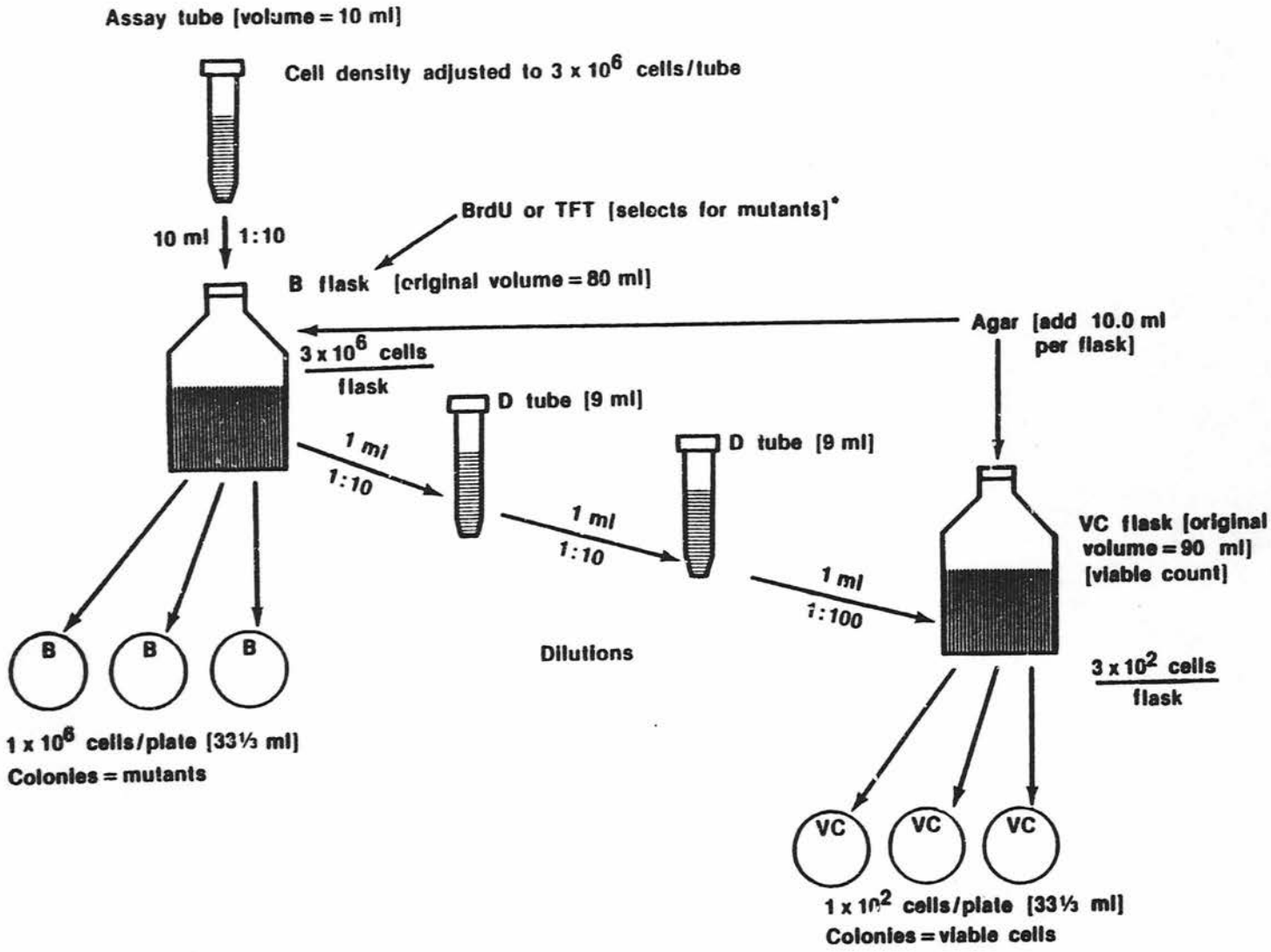
A 9,000 x g supernatant prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) is purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

6. REFERENCE

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res., 31:17-29, 1975.



*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

1) The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.

2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.

3) The average negative control suspension growth factor should not be less than about 12. The optimal value is 25, which corresponds to 5-fold increases in cell number for each of the two days following treatment of the experimental cultures.

4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 5×10^{-6} to 50×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.

5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5 µl/ml EMS (nonactivation assay) is 300 to 800 x 10^{-6}; for 0.3 µl/ml DMN (activation assay) the normal range is 200 to 800 x 10^{-6}. The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than 2.5 x 10^6 cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.

9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.

ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least 10×10^{-6}. The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.

- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.

- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test material will be evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10 µl/ml) for water-soluble materials or 1 mg/ml (or 1 µl/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be evaluated as nonmutagenic in this assay system.

The ASSAY ACCEPTANCE AND EVALUATION CRITERIA are presented to acquaint the sponsor with the considerations used by the study director to determine assay validity and the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20989 LBI Assay No. 5124

TYPE of STUDY Mouse Lymphoma Forward Mutation Assay

This final study report was reviewed by the LBI Quality Assurance Unit on October 22, 1980. A report of findings was submitted to the Study Director and to Management on October 24, 1980

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Patrice M. Cuccaro
Auditor, Quality Assurance Unit

Litton BIONETICS

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